Olive Oil and Red Wine Antioxidant Polyphenols Inhibit Endothelial Activation

Antiatherogenic Properties of Mediterranean Diet Phytochemicals

Maria Annunziata Carluccio, Luisa Siculella, Maria Assunta Ancora, Marika Massaro, Egeria Scoditti, Carlo Storelli, Francesco Visioli, Alessandro Distante, Raffaele De Caterina

Objective—Epidemiology suggests that Mediterranean diets are associated with reduced risk of cardiovascular disease. Because monocyte adhesion to the endothelium is crucial in early atherogenesis, we evaluated whether typical olive oil and red wine polyphenols affect endothelial–leukocyte adhesion molecule expression and monocyte adhesion.

Methods and Results—Phytochemicals in olive oil and red wine, including oleuropein, hydroxytyrosol, tyrosol, elenolic acid, and resveratrol, with or without antioxidant activity, were incubated with human umbilical vein endothelial cells for 30 minutes, followed by co-incubation with bacterial lipopolysaccharide or cytokines to trigger adhesion molecule expression. At nutritionally relevant concentrations, only oleuropein, hydroxytyrosol, and resveratrol, possessing a marked antioxidant activity, reduced monocytoid cell adhesion to stimulated endothelium, as well as vascular cell adhesion molecule-1 (VCAM-1) mRNA and protein by Northern analysis and cell surface enzyme immunoassay. Reporter gene assays with deletional VCAM-1 promoter constructs indicated the relevance of nuclear factor-κB, activator protein-1, and possibly GATA binding sites in mediating VCAM-1 transcriptional inhibition. The involvement of nuclear factor-κB and activator protein-1 was finally demonstrated at electrophoretic mobility shift assays.

Conclusions—Olive oil and red wine antioxidant polyphenols at nutritionally relevant concentrations transcriptionally inhibit endothelial adhesion molecule expression, thus partially explaining atheroprotection from Mediterranean diets. **(*Arterioscler Thromb Vasc Biol*. 2003;23:622-629.)**

Key Words: atherosclerosis ■ adhesion molecules ■ VCAM-1 ■ polyphenols ■ Mediterranean diets

Diet is a cornerstone of cardiovascular disease prevention.[1] Mediterranean diets are associated with a low incidence of atherosclerotic disease,[2,3] but data about the specific dietary constituents involved and mechanisms conferring cardioprotection are still sparse. There is consistent evidence for an antioxidant activity of some selected phenolic compounds from olives and grapes both in vitro[4,5] and in vivo.[6,7] *Trans*-resveratrol is a constituent of the skin of grapes, its concentration reaches 1.5 to 7 mg/L in red wine.[8] Oleuropein and hydroxytyrosol are present in a particularly high concentration in extra virgin olive oil (50 to 800 mg/kg)[9] and in olives (about 2 g/100g of dry weight).[10] Hydroxytyrosol is present also in the byproducts of olive oil production, where it may show biological properties preventing passive smoking-induced oxidative stress.[11] These compounds show several antiatherogenic activities, such as the inhibition of LDL oxidation,[12–14] platelet aggregation,[15,16] and the endothelial expression of tissue factor.[17] Moreover, olive oil polyphenols (600 ppm) added to virgin olive oil show protective effects in inflammation models in vivo,[18] and red wine polyphenols inhibit vascular smooth muscle cell migration[19] and enhance the expression and activity of endothelial nitric oxide synthase.[20]

Local leukocyte recruitment into the vessel wall is an early step in atherogenesis and it is largely explained by the increased expression of endothelial leukocyte adhesion molecules.[21] Because the transcriptional activation of adhesion molecules is sensitive to the intracellular redox status,[22,23] we investigated the effects of olive oil and red wine polyphenols on monocyte adhesion and the expression of endothelial leukocyte adhesion molecules as well as some potential mechanisms involved.

Methods

Reagents

Hydroxytyrosol was synthesized according to Capasso et al.[24] Oleuropein aglycone was obtained from oleuropein glycoside after enzymatic digestion.[25] Tyrosol was purchased from Sigma. Elenolic acid was isolated from extra-virgin olive oil by reverse-phase high performance liquid chromatography.[26] Oleuropein glycoside was obtained from Extrasynthèse (Genay, France), and resveratrol from Sigma (Figure 1). Resveratrol, hydroxytyrosol, tyrosol, and elenolic

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From the C.N.R. Institute of Clinical Physiology (M.A.C., M.A.A., M.M, E.S., A.D.), Lecce; Department of Biology (L.S., C.S.), University of Lecce; Institute of Pharmacological Sciences (F.V), School of Pharmacy, University of Milan; and Chair of Cardiology (R.D.C.), "G. d'Annunzio" University, Chieti, Italy.

Correspondence to Raffaele De Caterina, MD, PhD Chair of Cardiology, "G. d'Annunzio" University - Chieti, c/o Ospedale S. Camillo de Lellis, Via Forlanini, 50, I-66100 Chieti, Italy. E-mail rdecater@unich.it

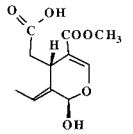
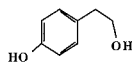
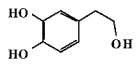
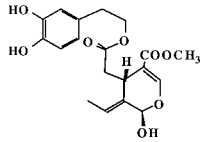
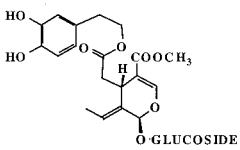
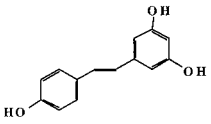
Structure	Name	Solubility	Antioxidant Properties
	**Elenolic Acid** 3-Formyl-3,4-dihydro-5-(methoxycarbonyl)-2-methyl-2H-pyran-4-acetic acid	Ethanol	None (unpublished data)
	**Tyrosol** 4-hydroxy-phenyl-ethanol	Ethanol/water	None [4]
	**Hydroxytyrosol** 2-(3,4-dihydroxy-phenyl)ethanol	Ethanol/water	Strong antioxidant [4]
	**Oleuropein aglycone**	Ethanol	Strong antioxidant [4]
	**Oleuropein glycoside** 2-(3,4-dihydroxyphenyl)ethyl(2S-(2alpha,3E,4beta))-3-ethyllidene-2-(beta-D-glucopyranosyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetate	Water	Strong antioxidant [4]
	**Resveratrol** 3,4'5- trihydroxy-*trans*-stilben	Ethanol	Strong antioxidant [14]

Figure 1. Structure, name, and physicochemical characteristics of the compounds used.

acid were dissolved in ethanol; oleuropein aglycone in methanol or ethanol; and oleuropein glycoside in water.

Interleukin (IL)-1β and tumor necrosis factor (TNF)-α were obtained from Genzyme, Cambridge, MA. Lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma, as well as all other reagents when not otherwise specified.

Endothelial Cell Cultures

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs) were harvested and maintained as described previously.[27,28] HUVECs and BAECs were grown in the presence of 10% fetal bovine serum and formed a confluent monolayer of polygonal cells expressing von Willebrand factor, as determined by their content of immunoreactive protein. Once grown to confluence, HUVECs and BAECs were replated on 1.5% gelatin-coated flasks at 20, 000 cells/cm^2, and used within passage 4.

Before treatments, confluent cells were shifted to media containing 4% fetal bovine serum, incubated in the absence (vehicle) or presence of varying concentrations (0 to 100 μmol/L) of each polyphenol for 0 to 2 hours, and then co-incubated with vehicle or polyphenols in the presence of LPS, cytokines (IL-1β, TNF-α), or PMA for additional 4 to 16 hours.

Detection of Cell Surface Molecules

Assays of cell surface molecules were conducted by cell surface enzyme immunoassay (EIA), using primary mouse antihuman monoclonal antibodies against VCAM-1 (Ab E1/6), E-selectin (Ab H18/7), intercellular adhesion molecule-1 (ICAM-1; HU5/3), or the monoclonal antibody E1/1, recognizing a constitutive and noncytokine-inducible endothelial cell antigen,[29] as previously described.[27] Primary antibodies were obtained from hybridoma supernatants, kindly provided by Michael A. Gimbrone (Harvard Medical School, Boston, MA).

Assessment of Cell Number and Viability

Cell number was assessed by direct cell counting of adherent cells, after trypsine detachment, in a Neubauer hemocytometer (VWR Scientifics), and stained by Trypan blue. The percentage of cells excluding Trypan blue was taken as a measure of cell viability.

Monocytoid Cell Adhesion Assays

Monocytoid U937 cells were obtained through American Tissue Culture Collection (Rockville, MD) and grown in RPMI medium 1640 (Gibco BRL, Gaithersburg, MD) containing 10% FCS. For the adhesion assays, HUVECs were grown to confluence in 6-well tissue culture plates, after which LPS or TNF-α was added for an additional 16 hours to induce the expression of VCAM-1, in the presence or absence of polyphenols (1 to 100 μmol/L). For control, some monolayers were treated with a mouse antihuman monoclonal antibody (E1/6) against VCAM-1. Adhesion assays were performed by adding 1 mL of the concentrated U937 cell suspension to each

monolayer under rotating conditions (63 rpm) at 21°C, as described.[27]

Isolation of RNA and Northern Analysis

Endothelial cells were pretreated for 30 minutes with polyphenols followed by a 4-hour stimulation with LPS (1 μg/mL) or TNF-α (10 ng/mL). Northern analysis was performed as described.[28]

Transfection Assays

Human VCAM-1 promoter constructs containing the chloramphenicol acetyltransferase (CAT) reporter gene were described previously by Neish et al.[30] Bovine aortic endothelial cells were transfected with each reporter plasmid (20 μg) using the calcium phosphate precipitation method. As an internal control for transfection efficiency, pRSV β-galactosidase (β-GAL) plasmid (5 μg) was co-transfected in all experiments. Cells (60% to 70% confluent) were stimulated 48 hours after transfection with LPS (1 μg/mL) or TNF-α (10 ng/mL) with or without pretreatment with polyphenols (15 μmol/L for 30 minutes), and cellular extracts prepared 15 hours later. Transfections and assays for CAT and β-GAL were performed as described previously.[28]

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides containing binding sequences for nuclear factor-κB (NF-κB) and activator protein- 1 (AP-1) present in the VCAM-1 promoter and corresponding oligonucleotide mutants were from Gibco BRL, and poly(dI-dC) from Pharmacia Biotech (Piscataway, NJ). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Melville, NY).

Confluent HUVECs were pretreated for 30 minutes with 0 to 100 μmol/L polyphenols and then exposed to LPS (1 μg/mL) or TNF-α (10 ng/mL) for 1 hour. Cells were scraped mechanically and nuclear extracts prepared as described.[28] Wild-type oligonucleotide probes from the human VCAM-1 promoter were synthesized to encompass the two NF-κB binding sites (underlined) described at coordinates −77 and −63 (5′-CTGCCCTGGGTTTCCCCTTGAAGGGATTTCCCTCC-3′) and the AP-1 binding site (underlined) located at −490 from the transcription starting site (5′-TTCCGGCTGACTCATCAAGCG-3′).[30]

Oligonucleotides probes were radiolabeled by Klenow filling-in as described.[27] The DNA binding reaction was performed at 30°C for 15 minutes in a volume of 20 μL containing 8 μg of nuclear extracts.[28] Samples were subjected to electrophoresis on native 5% 0.5× TRIS–borate–polyacrylamide gels. Specificity of the assay was determined by including a 50- to 100-fold excess of unlabeled competing wild-type or mutant sequences in the binding mixture. The mutant oligonucleotides for NF-κB (5′-CTGCCCTGAGTCACGCCTTGAAGAGACATCACTCC-3′) and AP-1 (5′ TGGCGGCTCCATGGTCAAGCG-3′) contain four nucleotide mutations in each binding site. After electrophoresis, gels were dried and directly autoradiographed using Kodak X-AR films.

Statistics

Two-group comparisons were performed by the Student *t* test for unpaired values. Comparisons of means of ≥3 groups were performed by ANOVA, and the existence of individual differences, in case of significant *F* values at ANOVA, tested by Scheffé's multiple contrasts.

Results

Structurally Unrelated Polyphenols Inhibit Stimulated VCAM-1 Expression

Among olive oil and red wine polyphenols tested, oleuropein, hydroxytyrosol, and resveratrol significantly inhibited LPS-stimulated expression of VCAM-1 in a concentration-dependent fashion as assessed by cell surface EIA (Figure 2A). The IC_{50} was around 30 μmol/L for resveratrol and 15 μmol/L for oleuropein aglycone, this last being more effective than its corresponding glycoside analogue. No significant effects on adhesion molecule expression were obtained, in our experimental system, with the two other tested phytochemicals from olive oil, elenolic acid, and tyrosol. The efficacy of oleuropein, hydroxytyrosol, and resveratrol ap-

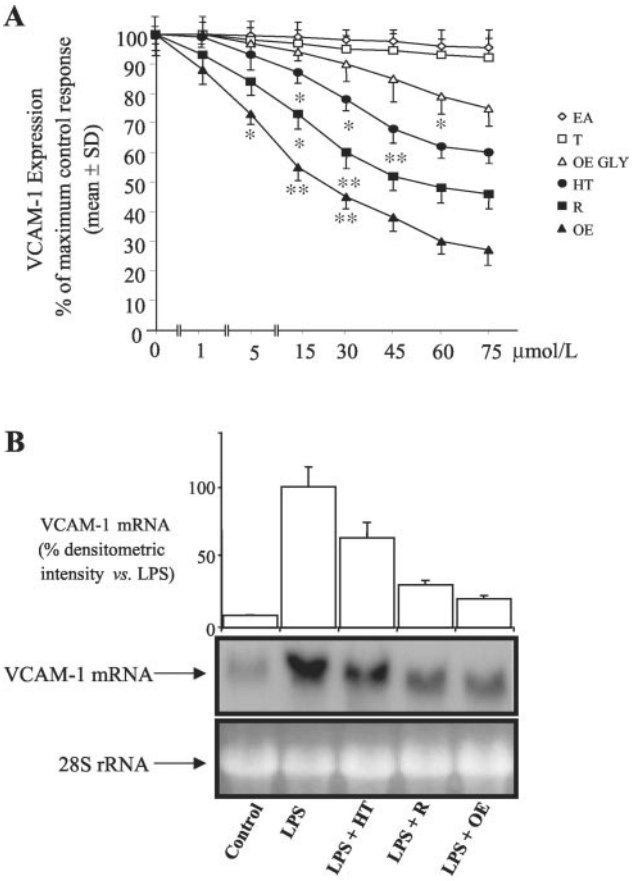


Figure 2. Structurally unrelated polyphenols inhibit LPS-stimulated VCAM-1 expression at protein and mRNA levels. A, Olive oil and red wine antioxidant polyphenols concentration-dependently inhibit LPS-stimulated VCAM-1 expression in HUVECs, as assessed by cell surface EIA. Compounds were added to tissue culture medium for 30 minutes at various concentrations, as indicated, after which VCAM-1 expression was induced by the addition of LPS at 1 μg/mL for a further 16 hours. All data have been plotted as percentage of maximum control response (% of stimulated response without polyphenols). Control vehicles (ethanol 0.05% vol/vol or methanol 0.005% vol/vol) had no effect on the stimulated expression of VCAM-1. Data are based on 6 different experiments, each consisting of ≥8 repeats for each condition. *$P<0.05$ and **$P<0.01$ versus LPS alone. B, Polyphenols decrease steady-state VCAM-1 mRNA levels at low concentrations (15 μmol/L). HUVECs were treated with polyphenols for 30 minutes, followed by stimulation with LPS for 4 hours. Isolated total RNAs (20 μg/lane) were examined by Northern analysis for VCAM-1 mRNA. The bottom panel shows 28S rRNA, stained by ethidium bromide, as a control for equal loading of lanes. Northern analysis is shown in the middle panel. Quantitative analysis by densitometry of specific bands is shown in the top panel, where the intensity of each band is expressed as percent of maximum control response to LPS. This blot is representative of a series of 4 similar ones (4 separate experiments) run in similar conditions.

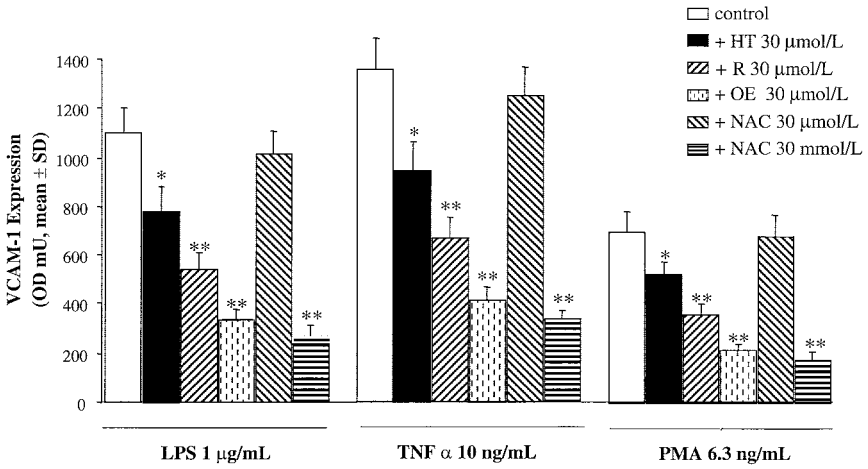


Figure 3. Effects of olive oil and red wine antioxidant polyphenols on VCAM-1 expression are independent of stimuli used to elicit endothelial activation. Olive oil and wine antioxidant polyphenols similarly reduce VCAM-1 expression induced by LPS, TNF-α, or PMA in HUVECs as assessed by cell surface EIA. Antioxidants were all added 30 minutes before the addition of the stimulus. The antioxidant NAC was devoid of any effect at 30 μmol/L whereas it significantly inhibited VCAM-1 expression at 30 mmol/L. OD mU denotes milliunits of optical density. Data are based on 4 different experiments, each consisting of $\geq$8 repeats for each condition. *$P<0.05$ and **$P<0.01$ vs LPS, TNF-α, or PMA alone.

pears strictly related with their antioxidant activity, tyrosol or elenolic acid being devoid of antioxidant activity (Figure 1).

The phytochemicals tested at concentrations used ($<$100 μmol/L) did not produce cellular toxicity, as assessed by cell number and viability (ie, morphology and Trypan blue exclusion), and were specific for proteins expressed during endothelial activation because they did not affect the expression of the constitutive endothelial surface antigen E1/1 (data not shown).

Olive Oil and Red Wine Antioxidant Polyphenols Decrease VCAM-1 mRNA Levels

To obtain some preliminary insight on the mechanism(s) by which olive oil and red wine antioxidant phytochemicals affect endothelial activation, we investigated their effects on LPS-stimulated VCAM-1 mRNA steady-state levels. Northern analysis demonstrated a clear decrease in VCAM-1 mRNA levels on incubation with phenolic compounds possessing activity on VCAM-1 protein expression (Figure 2B). Densitometric analysis of autoradiographic bands showed a reduction of around 60%, 40%, and 25% versus LPS alone for oleuropein aglycone, resveratrol, and hydroxytyrosol, respectively. These results are in good agreement with the reduction in protein expression (Figure 2A) and indicate that reduction of LPS-stimulated VCAM-1 expression by tested compounds occurs at a pretranslational level. Effects of antioxidant polyphenols on VCAM-1 expression are independent of stimuli used to elicit endothelial activation

We assessed and compared polyphenol inhibition of VCAM-1 expression in response to structurally unrelated agonists such as LPS, TNF-α, and PMA, this last used as a stimulus for endothelial activation that bypasses membrane receptors. Antioxidant polyphenols inhibited VCAM-1 expression to the same extent with all stimuli and independent of the relative potency of stimuli (Figure 3). Similar results were obtained using IL-1β (not shown). Because of this proven similarity of effects with various stimuli for endothelial activation, LPS was used in most experiments performed.

We also compared the inhibitory effect of antioxidant phytochemicals with the inhibitory effect of *N*-acetyl cysteine (NAC), a well-known antioxidant reported to efficiently reduce the expression of several endothelial adhesion molecules.[23] NAC suppressed VCAM-1 expression at 30 mmol/L in response to various stimuli but was unable to significantly modulate the expression of VCAM-1 at concentrations 1000 times lower, at which olive oil and red wine antioxidant polyphenols were active (Figure 3).

Olive Oil and Red Wine Antioxidant Polyphenols Suppress VCAM-1 Promoter Activity and the Activation of Transcription Factors NF-κB and AP-1

To determine whether olive oil and red wine antioxidant polyphenols regulate VCAM-1 promoter and to identify promoter regions involved, we transfected BAECs with deletional VCAM-1 promoter constructs linked to the chloramphenicol acetyltransferase reporter gene.[30] F0.CAT is the functional VCAM-1 promoter containing AP-1, GATA, and two tandem κB sites (Figure 4A). F3.CAT contains the NF-κB binding sites without GATA or AP-1 (Figure 4A). F4.CAT only contains a TATA box (Figure 4A). TNF-α increased F0.CAT activity by 8-fold and F3.CAT activity by 4-fold compared with unstimulated cells (Figure 4B), indicating the relevance of the AP-1 and GATA sites in potentiating the two tandem NF-κB sites to elicit TNF-α–induced transcription. Pretreatment with olive oil and red wine antioxidant polyphenols (15 μmol/L, 30 minutes), inhibited TNF-α–stimulated promoter activity by 70% to 80% in F0.CAT and by 40 %to 50% in F3.CAT transfections (Figure 4B). Similar results were obtained using LPS as the stimulus (not shown). These results suggest that inhibition of VCAM-1 expression by olive oil and red wine polyphenols is transcriptional and likely the result of a modulation of different transcription factors, mainly NF-κB, but also AP-1 and possibly GATA.

Because transcription factors NF-κB and AP-1, which contain binding sites in the 5′-flanking regions of the VCAM-1 promoter, are known to be redox sensitive because their activation is inhibited by antioxidants,[22,31–33] we sought to determine whether olive and red wine antioxidant polyphenols actually inhibit the activation of these transcription factors, thus providing a likely explanation for the inhibition of VCAM-1 transcription. To this purpose, we performed gel shift assays using radiolabeled oligonucleotides corresponding to the tandem κB and AP-1 sites on the VCAM-1 promoter. We observed that polyphenols possessing antioxi-

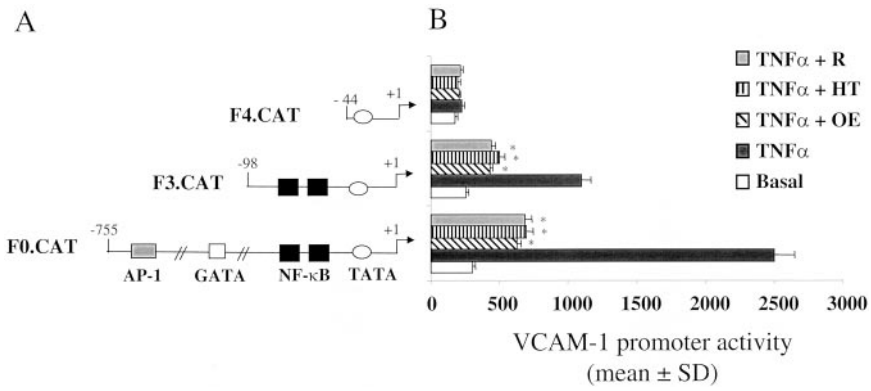


Figure 4. Effects of olive oil and red wine antioxidant polyphenols on VCAM-1 promoter activity. A, Structure of the promoter constructs used, showing the binding sites for transcription factors and the distance from transcription starting site. B, Plot of VCAM-1 promoter activity with the various promoter constructs used, in the absence or presence of TNF-α (10 ng/mL) $\pm$ the various antioxidant polyphenols used (all at 15 μmol/L). Promoter activity is expressed as mean$\pm$SD of the CAT/β-GAL ratio of 3 replicates for each condition. *$P<0.01$ vs TNF-α alone. The experiment shown is representative of a series of 3 similar ones with identical results.

dant activity, at 15 μmol/L, decrease the amount of the shifted complex induced by LPS, corresponding to NF-κB (Figure 5A). Densitometric analysis indicated that oleuropein aglycone, *trans*-resveratrol, and hydroxytyrosol inhibit the activation of NF-κB by 70%, 60%, and 50%, respectively.

Treatment with olive oil and wine antioxidant polyphenols also affected the induced activation of AP-1. The intensity of shifted band was decreased by 50%, 40%, and 30% by oleuropein aglycone, hydroxytyrosol, and *trans*-resveratrol, respectively (Figure 5B).

Olive Oil and Red Wine Antioxidant Polyphenols Are Global Inhibitors of Endothelial Activation

We investigated the effects of polyphenols on other LPS-inducible endothelial leukocyte adhesion molecules, such as E-selectin and ICAM-1. Similarly to VCAM-1, the induced expression of E-selectin (Figure 6A) and ICAM-1 (Figure 6B) was also reduced by all tested polyphenols in a concentration-dependent fashion. Oleuropein aglycone was again the most active polyphenol in inhibiting the stimulated expression of both adhesion molecules, already at concentrations of 5 μmol/L. These results indicate a generalized effect of these natural polyphenols on endothelial activation.

Olive Oil and Red Wine Antioxidant Polyphenols Decrease Monocytoid Cell Adhesion to HUVECs

To evaluate the functional consequences of olive oil and red wine polyphenol-induced reduction in the expression of adhesion molecules, we tested whether their incubation with HUVECs affected the adherence of human monocytoid U937 cells to HUVECs. Monocytoid cells did not adhere to unstimulated HUVEC monolayers (control) but adhered to a great extent to LPS-stimulated HUVECs (Figure 7). This stimulated adhesion was clearly inhibited (by >50%) by the anti-VCAM-1 monoclonal antibody E1/6 (not shown). To a degree comparable to the inhibition of endothelial leukocyte

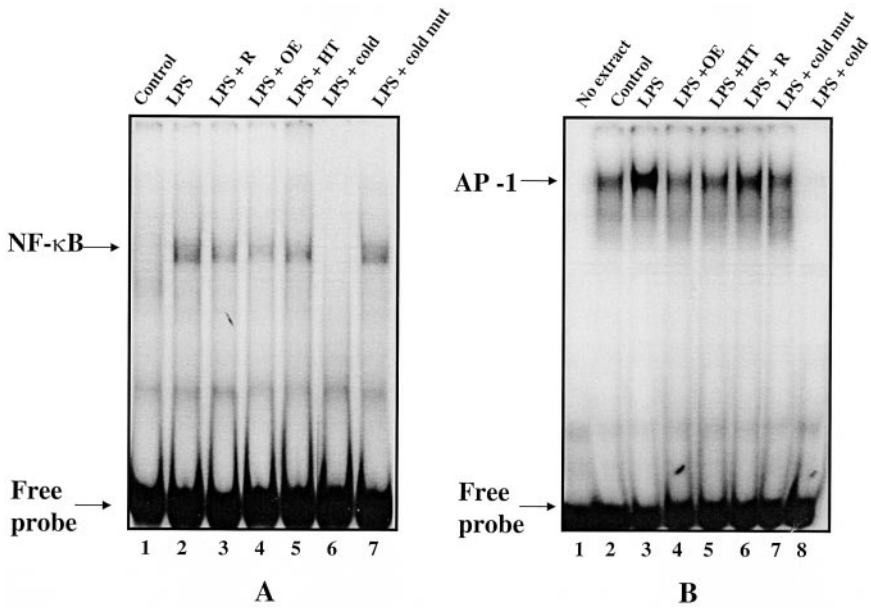


Figure 5. Olive oil and red wine antioxidant polyphenols inhibit LPS-triggered NF-κB and AP-1 activation at EMSA. A, Antioxidant polyphenols decrease NF-κB activation. Compared with lane 1, with nuclear extracts obtained in control, unstimulated conditions, a retardation of electrophoretic mobility (gel shift) of the ^{32}P-labeled oligonucleotide containing the VCAM-1 NF-κB sequence is seen in lane 2, obtained with nuclear extracts from cells stimulated with LPS, indicating the formation of DNA-protein complexes. The intensity of this band, proportional to the amount of transcription factor translocated into the nucleus and binding to DNA, is strongly reduced on treatment of endothelial cells with antioxidant polyphenols (15 μmol/L) before stimulation (lanes 3 to 5). Equal amounts of nuclear proteins (4 to 6 μg) were used for the binding assay. Specificity of the retarded band for NF-κB, not present in nuclear extracts in unstimulated conditions and only appearing in stimulated conditions, is demonstrated by its total abolition with a 100-fold molar excess of cold wild-type oligonucleotide (lane 6), and its preservation with a similar excess of mutant oligonucleotide (lane 7). This EMSA is representative of a series of 3 similar ones. B, Antioxidant polyphenols decrease AP-1 activation. The EMSA shows a reduced activation of AP-1 by antioxidant polyphenols (15 μmol/L) in HUVECs stimulated with LPS (1 μg/mL) for 1 hour (lanes 4 to 6) versus HUVECs stimulated with LPS alone (lane 3). Specificity of the shifted band for AP-1 is confirmed by its total abolition with a 100-fold molar excess of cold wild-type oligonucleotide (lane 8), and its preservation with a similar excess of mutant oligonucleotide (lane 7). This EMSA is representative of a series of 3 similar ones.

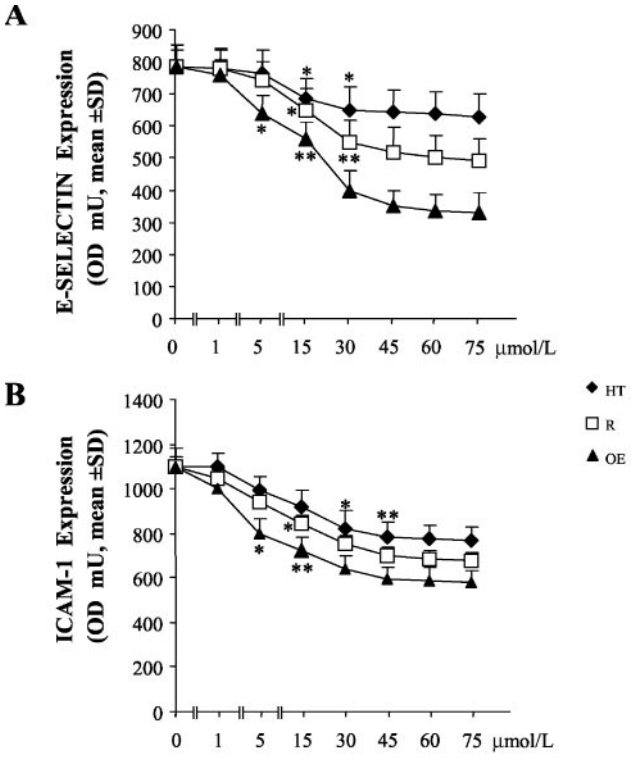


Figure 6. Olive oil and red wine antioxidant polyphenols are global inhibitors of endothelial activation, affecting the expression of various adhesion molecules. HUVECs were treated with different concentration of polyphenols for 30 minutes and then stimulated with LPS. Adhesion molecule expression was assessed by cell-surface EIA by specific monoclonal antibodies, as described in text. Data are based on 5 different experiments, each based on ≥8 repeats for each condition. *$P<0.05$ and **$P<0.01$ vs LPS alone.

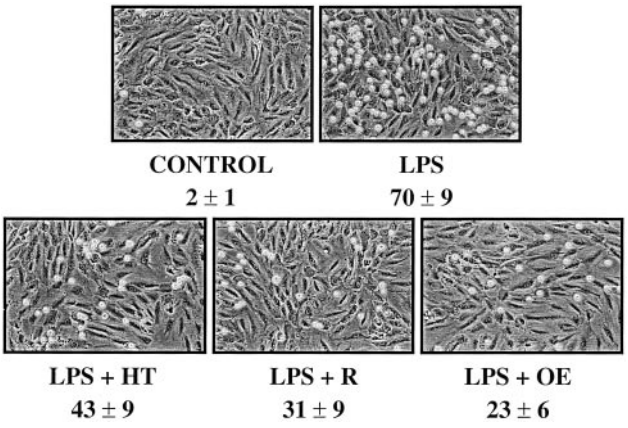


Figure 7. Olive oil and red wine antioxidant polyphenols decrease monocytoid cell adhesion to HUVECs. U937 cells, added in suspension to HUVECs as described in the Methods section, do not normally adhere to unstimulated monolayers, and are easily removed with washing (control, no stimulation), whereas adhesion is dramatically increased by the addition of LPS (1 µmol/L). Polyphenols (all at 15 µmol/L) decrease U937 cell adhesion by >50% (lower panels). Labels below the photographs show cell counts (mean±SD, n=6 for each condition) within a grid area at high power field (0.16 mm²). This experiment was repeated 4 times with similar results. HT, hydroxytyrosol; R, resveratrol; OE, oleuropein aglycone.

adhesion molecule expression, treatment of HUVECs with antioxidant polyphenols (15 µmol/L) significantly inhibited LPS-induced monocyte adhesion (Figure 7).

Discussion

This study shows the anti-inflammatory and therefore possibly antiatherogenic activity of some phenolic compounds from olive oil and red wine. We demonstrated the inhibition of both the stimulated expression of VCAM-1 and of monocyte adhesion to human vascular endothelial cells. The effects of antioxidant polyphenols also apply to E-selectin and ICAM-1, suggesting an action on a common intracellular pathway of endothelial activation. Most relevant, such effects occurred at low micromolar concentrations within the concentration range expected after nutritional intake from Mediterranean diets[34]. It is likely that such beneficial effects would be amplified in vivo because of the continuous exposure of vascular endothelia to these compounds and a possible additive or more than additive effect of various co-administrated compounds. Preliminary experiments of ours indeed suggest at least additive effects for their co-administration, but this issue clearly deserves further investigation.

Among the tested molecules, oleuropein aglycone and hydroxytyrosol were the most potent phytochemicals in reducing the expression of adhesion molecules. This is consistent with their orthodiphenolic structure, which confers strong antioxidant properties.[35] A more efficient anti-inflammatory role of the aglyconic, compared with the glycosidic, form of oleuropein possibly derives from the greater lipophilicity of the former, a property that should allow better cell membrane incorporation and/or interaction with other lipids.[36] Also of interest, however, is the evidence that, in comparison with other effective and well-known antioxidants, such as NAC, the tested antioxidant polyphenols achieved the same extent of inhibition at concentrations about 1000-fold lower. Such comparisons are revealing an unexpected heterogeneity among different antioxidants with respect to endothelial activation, which deserves further investigation.

The inhibition by antioxidant polyphenols of VCAM-1 expression induced by different agonists (including PMA) strongly argues that antioxidant polyphenols act downstream of any membrane receptor, at a step common to all agents. Being the gene of VCAM-1 transcriptionally regulated,[37] we first examined the activity of effective polyphenols on steady-state VCAM-1 mRNA, which was reduced to an extent paralleling protein expression. This indicates that a pretranslational action of these compounds fully explains the inhibition of VCAM-1 expression and its functional consequences on monocytoid cell adhesion.

The VCAM-1 promoter contains various binding sites for transcription factors, such as NF-κB, AP-1, and GATA.[30] Transfection studies using various VCAM-1 gene promoter constructs showed that antioxidant polyphenols from olive oil and red wine repressed VCAM-1 gene transcription. Because inhibition of promoter activity was decreased by deletion of binding sites for AP-1 and GATA and totally abrogated by

deletion of the two κB sites, an interference by antioxidant polyphenols with redox-sensitive nuclear transcription factors NF-κB and AP-1[37,38] was logically suspected. This was confirmed by specific EMSA, showing reduced activation of both NF-κB and AP-1, the interaction of which is known to amplify VCAM-1 promoter activation.[39] It seems therefore most likely that their simultaneous inhibition results in at least additive atheroprotective effects.

Although our results are the first to quantitatively compare the effects of various antioxidant dietary polyphenols on endothelial leukocyte adhesion molecules, they are in agreement with previous reports on resveratrol-induced effects on endothelial activation,[40,41] including suppression of TNF-α–induced activation of NF-κB and AP-1.[42] Here, an interference by resveratrol with TNF-α–induced activation of mitogen-activated protein kinase kinase and c-Jun N-terminal protein kinase was shown, interpreted as a consequence of the reduced generation of reactive oxygen species and lipid peroxidation.[42] Whether this also occurs for other antioxidant polyphenols and with respect to inhibition of adhesion molecule expression remains to be demonstrated.

In conclusion, our findings reveal new molecular mechanisms by which several quantitatively minor components of the Mediterranean diet may prevent early atherogenesis. Together with fatty acids,[27,43] they are among the first examples of how selected nutrients may directly regulate the expression on proinflammatory/proatherogenic genes.

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